Anionic salivary proteins associated with connective tissue disorders: sialated tissue kallikreins

D GREAVES,¹ J T WHICHER,⁴ K D BHOOLA,² C SCULLY,¹ S FLINT,¹ S R PORTER,¹ R E CHAMBERS,³ P A MADDISON,⁵ J A BEELEY,⁶ AND R MATTHEWS¹

From the Departments of ¹Oral Medicine, ²Pharmacology, and ³Chemical Pathology, University of Bristol, Bristol, UK; the ⁴Department of Chemical Pathology, University of Leeds, Leeds, UK; ⁵Royal Hospital for Rheumatic Disease, Bath; and the ⁶Oral Biochemistry Unit, University of Glasgow Dental School, Glasgow

SUMMARY Parotid saliva was collected from 32 patients with rheumatoid arthritis, 10 with systemic lupus erythematosus, three with mixed connective tissue disease, 12 with progressive systemic sclerosis, two with primary Sjögren's syndrome, and four with Raynaud's syndrome. Tissue kallikreins were measured by radioimmunoassay, and saliva samples were subjected to isoelectric focusing followed by immunoblotting or silver staining. The results showed that the saliva of patients with connective tissue diseases contained increased amounts of immunoreactive tissue kallikrein. In addition, there was an increase in the multiple forms of anionic tissue kallikreins, resulting mainly from a shift in their distribution towards that of higher sialic acid content and lower isoelectric point. These changes were most obvious in patients with systemic lupus erythematosus. Novel or unusual glycosylation may explain the occurrence of increased amounts of anionic salivary proteins in connective tissue diseases.

A frequent complication of connective tissue diseases is secretory failure of exocrine glands, including the lacrimal and salivary glands, leading to dry eyes and dry mouth. This is known as secondary Sjögren's syndrome¹ and is often associated with rheumatoid arthritis, systemic lupus erythematosus, progressive systemic sclerosis, and mixed connective tissue disease.² Primary Sjögren's syndrome has no associated connective tissue disorder and differs from the secondary form clinically, serologically, and genetically.

A number of studies, using electrophoretic techniques of varying resolution, have shown that the saliva from patients with Sjögren's syndrome contains increased amounts of several anionic proteins.^{3–6} Furthermore, these proteins appear more frequently in the secondary form of the disease, and have been described in the saliva of patients with rheumatoid arthritis but without clinical evidence of salivary gland involvement.⁷ Although these anionic salivary proteins have been

subjected to concerted investigations, their identity has remained obscure. The initial aim of this study was to identify and characterise these proteins, and to ascertain whether they were more prevalent in a group with a particular connective tissue disease. By chance, we discovered that the dominant group of these anionic proteins in connective tissue diseases were tissue kallikreins (E.C.3.4.21.35), which are normally present in saliva, and whose anionic nature is conferred by their sialic acid composition.

Tissue kallikreins are present in a wide variety of tissues.^{8,9} They are members of a multigene family of serine proteases known to cleave kininogen and precursors of hormones to produce biologically active peptides. Clearly, the exciting possibility of unusual or novel glycosylation of proteins like tissue kallikrein may be important in our understanding of the primary cellular defect in connective tissue diseases.

Patients and methods

PATIENTS

We studied 63 patients (female 45, male 18; mean age 53.5 years, range 18–82 years) with connective tissue diseases (32 rheumatoid arthritis, 10 systemic

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Correspondence to Dr K D Bhoola, Department of Pharmacology, Medical School, University of Bristol, University Walk, Bristol BS8 1TD, UK.

lupus erythematosus, 3 mixed connective tissue disease, 12 progressive systemic sclerosis, 2 primary Sjögren's syndrome, 4 primary Raynaud's syndrome), who were diagnosed as definite or classical connective tissue disease according to American Rheumatism Association criteria,¹⁰ and 45 healthy patients matched for age and sex as controls. Ten patients were taking no drugs while the remainder were taking two or more drugs. We classified these into non-steroidal anti-inflammatory (38 patients), steroids, immunomodulatory, and other (comprising diuretics, antihypertensives, antidepressants, etc) drug groups.

SAMPLE COLLECTION

Parotid saliva, stimulated with citric acid (1%), was collected by mild suction of saliva from the parotid duct orifice through silicone tubing into a plastic tube kept on ice. The collections were performed mid-morning as salivary secretory proteins show diurnal variation. Samples were frozen and stored at -20°C until assayed. Urine was collected at the same time as saliva from the patients with connective tissue diseases and from the healthy controls.

ISOELECTRIC FOCUSING

Samples were freeze dried and reconstituted to a protein content¹¹ of 6 µg/µl for isoelectric focusing, which was performed on polyacrylamide gels (4.8%) over a pH gradient of 2.5 to 6.0 using 25 µl samples.⁷ The gels were stained sequentially with Coomassie brilliant blue G250⁷ (45 minutes) then silver stain¹² after several washes (30 minutes) in deionised water. Gels were dried and wrapped in cellophane preserving sheets supported on Gelbond (Miles Scientific, Slough, UK) before scanning on a laser densitometer (Ultrascan, LKB Produkter, Bromma, Sweden). The intensity of bands shown on the silver stained electrophoretograms of pI 3.0-4.0 (determined using a surface electrode) was expressed as integrated density (the total absorbance of the band). The intergel variation in measured density of a single band was 9.4% (n=20).

HUMAN TISSUE KALLIKREIN: STANDARDS AND ANTISERA

Purified human tissue kallikrein¹³ of urinary origin (kindly provided by Professor M Moriya, Japan) was used as a standard in the radioimmunoassay¹⁴ and on the electrophoretograms both for silver staining and immunoblotting. Human urinary kallikrein was purified¹⁴ by silica gel absorption, gel filtration on Sephadex G-75, DEAE-Sephadex, and affinity chromatography, with the final step involving rapid gel filtration. The pure preparation

was heterogeneous consisting of three isoenzymes of pI 3.9, 4.0, and 4.2. Enzymic activity was equivalent to 4.6 units/mg protein (one unit hydrolyses 1 µmol of D-Val-Leu-Arg-pNA nitroanilide/min at pH 8.0 and 37°C). Human salivary kallikrein was purified by a modification of the method described by Lemon *et al.*¹⁵ After absorption of the enzyme to DEAE-cellulose and gel filtration through Sephadex G-100 it was chromatographed twice on DEAE-Sephadex A-50. The final step involved passage through CNBr-Sepharose 4B coupled to antihuman albumin. The specific enzymic activity of this preparation was 38 units/mg protein.

Polyclonal antibodies specific to human urinary and salivary kallikrein were raised in rabbits. Specificity of the antisera was determined by Ouchterlony gel diffusion, immunoblotting, and radioimmunoassay.¹⁴ In gel diffusion human urinary and salivary kallikrein showed complete immunological identity when reacted against both antisera. In addition, both human urinary and salivary kallikrein cross reacted in an identical manner with antisera raised to human urinary kallikrein by Dr C Vidossich, Faculty of Biological Sciences, Catholic University of Santiago, Chile and by Dr David Proud, Division of Clinical Immunology, Johns Hopkins University School of Medicine, Baltimore, USA. No reaction was observed with aprotinin, urokinase, plasminogen, thrombin, human albumin, or trypsin.

IMMUNOBLOTTING

Electroblotting was performed on 0.45 µm nitrocellulose (Transphor 2005, LKB-Produkter) in transfer buffer pH 8.3 (25 mM TRIS-HCl, 150 mM glycine, 20% methanol). Rabbit antisera (10 ml, 1/200 dilution) to IgA, IgM, IgG, secretory component, κ light chains, λ light chains, lysozyme, β_2 -microglobulin, α_1 -acid glycoprotein, C reactive protein, serum amyloid P component, C1 esterase inhibitor (Dakopatts, Copenhagen, Denmark), tissue kallikrein, and salivary acidic proline-rich proteins (kind gift of Professor A Bennick, Toronto) were used as the first stage of the unlabelled bridge technique. Swine antirabbit antiserum (10 ml, 1/400 dilution) formed the link with the rabbit antipeptide oxidase/peroxidase complex (10 ml, 1/400 dilution) (Dakopatts). The blot was stained with 3-amino-2-ethylcarbazole (stock solution: 1% 3-amino-2-ethylcarbazole in acetone, 25 ml 0.05 M sodium acetate buffer pH 5.0, 15 µl 30% H₂O₂). Immuno-blots were also prepared on saliva samples incubated with neuraminidase (Sigma, Poole, Dorset, UK) at a concentration of 1 mg/ml of saliva for 36 hours at 4°C. Kallikrein concentrations were measured before and after neuraminidase treatment to determine whether the radioimmunoassay was

affected by the sialic acid content of the acidic forms of the enzyme.

STATISTICAL ANALYSIS OF THE DATA

Cumulative frequency curves of the integrated density of bands in the pI 3.0–4.0 range for groups with connective tissue disease and control groups were fitted to the logistic expression¹⁶:

$$Y = \frac{X^P}{X^P + K^P}$$

where Y is the density, K the 50% value, and P the slope. The same expression was used to determine the distribution of tissue kallikrein values (Y = tissue kallikrein in ng/μg parotid salivary protein) in each group. In addition, the data were analysed by the non-parametric Mann-Whitney U test and band density compared with tissue kallikrein concentrations by linear regression analysis.

Results

Isoelectric focusing of parotid saliva over the pH range 2.5–6.0 showed a very large number of bands when stained with a sensitive silver stain (Fig. 1a). The proline-rich proteins,¹⁷ which show inconsistent staining characteristics on silver staining, dominate the pattern between pH 4.0 and 4.8. For this reason we chose to study the most anodal proteins in the pH range 3.0–4.0, which appeared on preliminary assessment to be present in increased amounts in some patients with connective tissue diseases.

Immunoblotting of the anionic bands with antisera to a wide range of human serum and salivary proteins (see 'Patients and methods') showed reactivity primarily against human tissue kallikrein. A complex pattern of 12 immunoreactive tissue kallikrein bands was seen. Six of those present in the 'anionic region' (pI 3.0–4.0) represented the bands whose intensity was measured after silver staining (Fig. 1b). A further six bands were present in the pI range 4.0–5.0 and three in the pI range 4.0–4.25. The intensity of these bands, which had not been clearly visualised on silver staining owing to the presence of several overlapping non-kallikrein bands, did not vary in parallel with those in the anionic region. Two faint bands reacting with anti- α_1 -acid glycoprotein were seen at pI 2.7. Treatment of saliva samples and purified tissue kallikrein with neuraminidase largely removed the anodal kallikrein bands and resulted in their shift to a higher pI value (Figs 1a and b).

Analysis of the silver stained bands (Fig. 1a), measured by laser densitometry, showed a clear increase in anionic proteins in the saliva of some

Fig. 1a

Fig. 1b

Fig. 1 (a) Silver stained, isoelectrically focused saliva samples before and after (n) treatment with neuraminidase. A = normal saliva with a low concentration of anionic bands (representing the region 2.0 to 4.0 integrated density units); B = normal saliva with a higher concentration of anionic bands (representing the region 7.0 to 8.0 integrated density units); C = saliva from a patient with rheumatoid arthritis and a high concentration of anionic bands (representing the region 14.0 to 16.0 integrated density units). For integrated density units refer to Fig. 2. (b) Salivary samples from a normal subject containing a high concentration of anionic bands (A) and a patient with systemic lupus erythematosus (B) immunoblotted with anti-tissue kallikrein before and after (n) treatment with neuraminidase.

Fig. 2 The distribution of integrated band density (groups of five densitometric units) in (a) patients with connective tissue disease and (b) normal control subjects. RA=rheumatoid arthritis (32 patients); PSS=progressive systemic sclerosis (12 patients); SLE=systemic lupus erythematosus (10 patients); M comprises patients with primary Sjögren's syndrome (two patients), primary Raynaud's syndrome (four patients), and mixed connective tissue diseases (three patients); C=controls. (The total disease group range is 1-79 units with a median of 27 units; in the control group the range is 1-54 units with a median of 15 units).

patients with connective tissue diseases when compared with normal controls (Fig. 2). These findings were examined statistically by Dr R B. Barlow.¹⁶ The reason for fitting the data to a logistic expression was to determine whether the control and connective tissue disease values represented single or multiple populations. As evident from Fig. 3 the cumulative frequency curve indicated the presence of at least two populations in the connective tissue disease values, in contrast with the single population in the control data. Such important information cannot be readily ascertained from single means and standard error of the mean. It seems that systemic lupus erythematosus shows the greatest prevalence of high intensity silver staining in the anodal region and progressive systemic sclerosis the least (see Fig. 2). The possible influence of treatment on this relation was excluded by comparing band intensity in those patients taking no drugs with those prescribed drugs, using a two sided Mann-Whitney test; no significant differences ($p < 0.2$) were observed between the two groups. We further compared each drug group with those taking no drugs and also found no significant differences.

Immunoreactive salivary tissue kallikrein concentrations, measured by radioimmunoassay, were significantly higher in the group with connective tissue diseases than in the controls (Fig. 4). Unlike the silver staining, none of the individual disease groups was significantly different from the others. Furthermore, there was no correlation between the tissue kallikrein concentrations and the intensity of the anodal bands either in the controls ($r = 0.561$) or the group with connective tissue disease ($r = 0.371$). Neuraminidase treatment did not alter the salivary tissue kallikrein concentrations measured by radioimmunoassay.

Fig. 3 Cumulative frequency curves obtained by integrating the density values for controls ($\Delta \dots \Delta$) and patients with connective tissue diseases ($\blacksquare \dots \blacksquare$). The logistic expression values for the controls ($n = 45$) are $K1 = 14.45/0.12$, $P1 = 2.88/0.08$ and for connective tissue diseases ($n = 63$) $K2 = 23.40/0.18$, $P2 = 1.88/0.08$. The connective tissue disease curve is shifted to the right, indicating that anionic bands of higher integrated density occur more commonly in this group, and the distribution indicates the presence of more than one population in this group. This finding is more clearly seen on the histogram in Fig. 2.

Fig. 4 Cumulative frequency curves for tissue kallikrein values in the controls (Δ Δ) and patients with connective tissue diseases ($\blacksquare$ — $\blacksquare$). The logistic expression values for the controls ($n=40$) are $K1=0.89/0.01$; $P=2.26/0.08$ and for connective tissue diseases ($n=53$) $K2=1.24/0.01$; $P=1.81/0.08$. Although the frequency distribution curve shows similar populations for both groups, the curve for connective tissue disease is significantly shifted to the right, indicating the occurrence of greater tissue kallikrein values and variance in the patients with connective tissue diseases. When the data were analysed by linear regression no significant correlation was evident between the tissue kallikrein values and the density of the anionic bands either in the normal group ($r=0.560$) or the group with connective tissue diseases ($r=0.377$). The tissue kallikrein values were: control group, mean 1.16 ng/g protein, range 0.18 – 4.68 ; connective tissue disease group, mean 1.89 , range 0.16 – 7.08 .

Samples of urine from a subgroup of eight patients and 11 controls were examined by isoelectric focusing and immunoblotting. Preliminary experiments indicated that there was no variation in intensity of anionic bands (pI 3.0–4.0), paralleling the results seen in saliva. The bands present in the pI 4.0–5.0 range were more intense in the urine samples than those observed in saliva.

Discussion

Using isoelectric focusing, sensitive silver staining, and laser densitometry, we have been able to show that there is a wide distribution in concentration of proteins forming multiple bands with a pI of between 3.0 and 4.0 in the saliva of both normal subjects and patients with connective tissue diseases. We have identified most of these protein bands as multiple forms of tissue kallikrein (E.C.3.4.21.35), which on treatment with neuraminidase acquired a more cathodal electrophoretic mobility owing to removal of sialic acid residues. Furthermore, most bands identified as tissue kallikrein have a lower pI

(3.0–4.0) than the bands previously described by Mairs and Beeley⁷ (pI 3.95–4.25). There is, however, some overlap and a particularly prominent band seen in their electrophoretograms at pI 3.95 is clearly evident on our separations as tissue kallikrein. The greater sensitivity of the silver staining technique has allowed us to visualise the more anodal proteins, which are not seen with the Coomassie blue staining technique. Immunoblotting suggests that most bands in the pI range 3.95–4.25 are tissue kallikreins, but that there are also proteins rich in proline,¹⁷ and rheumatoid factors as shown by Mairs and Beeley.⁷ The proline-rich proteins show variable staining using silver and for this reason we chose not to measure the bands with a pI greater than 4.0.

We have shown that the concentration of immunoreactive tissue kallikrein in the parotid saliva of patients with connective tissue diseases was significantly greater than in normal controls. In contrast, normal values have been published for enzymically active tissue kallikrein in parotid or submandibular saliva in patients with rheumatoid

arthritis.¹⁸ Several studies have stated that enzymically active tissue kallikrein in whole rheumatoid arthritis saliva was either normal or raised.^{19, 20} Such differences in concentrations are probably due to the lack of specificity in the synthetic substrates used to measure the active enzyme.

From our results it is evident that patients with rheumatoid arthritis, systemic lupus erythematosus, mixed connective tissue disease, and Raynaud's syndrome have a greater quantity of anionic tissue kallikrein in their saliva. Furthermore, this finding did not appear to be related to the degree of clinically overt Sjögren's syndrome. It is notable that patients with progressive systemic sclerosis have normal concentrations of anionic proteins. Of relevance is the observation that tissue kallikrein concentrations were decreased in patients with sarcoidosis, even though there was no clinical evidence of salivary gland involvement.²¹ Our failure to find a clear correlation between the increased anionic forms and altered exocrine gland function may reflect the presence of a glycosylating defect before the appearance of clinically noticeable symptoms. Subsequent progression of the disease may involve additional factors.

In view of the widespread cellular sites of localisation of the kallikrein group of serine proteases we examined the urine of a small group of patients and normal subjects. Urinary kallikrein is thought to originate mainly from the renal connecting tubule cells.²² Anionic kallikrein bands sensitive to neuraminidase were present in the urine at low concentrations in both normal subjects and patients. The absence of high intensity anionic bands in urine paralleling those in saliva suggests, however, that the multiple anionic forms of tissue kallikrein synthesised in the salivary duct cell are not expressed in the renal connecting tubule cells. In urine, kallikrein bands of pI 4.0-5.0 showed a greater intensity of staining than those in saliva. Differences in both structure and regulation of synthesis of salivary and urinary tissue kallikreins have been suggested on the basis of both immunochemical and pharmacological studies.²³

One explanation for the multiple anionic forms in parotid saliva may be the sequential removal of carbohydrate chains from the tissue kallikrein molecule by neuraminidase arising from desquamated inflammatory cells. The major component of human salivary kallikreins has a pI value of 3.9. Thus the unlikely hydrolysis of sialic acid residues by neuraminidase from inflammatory cells may only mask anionic forms of the enzyme with even lower pI values.

Normal carbohydrate microheterogeneity, due to the linking of different sialic acid residues to the

parent protein, has been well described in pig submandibular¹⁵ and pancreatic kallikrein,²⁴ and in rat²⁵ and human^{13, 26-28} urinary kallikrein. The presence therefore of increased amounts of anionic molecules of tissue kallikrein in the saliva of patients with connective tissue diseases suggests either that there is a primary genetic abnormality linking these diseases and certain anionic forms of kallikrein or that early disease within the gland causes a post-transcriptional change which modifies the carbohydrate side chain substitution of the kallikrein molecules. Whether such an alteration in the synthesis of unusual or novel acidic carbohydrate moieties could account for the secretion of low pI anionic kallikrein by salivary duct cells, and whether such a change has any relevance to the pathogenesis of connective tissue diseases, are open questions.

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